IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re: Application of Giltsu Choi, *et al.*	: Group Art Unit: 1655
Appln. No.: TBA	: Examiner: J. Einsmann
Filed: August 14, 2000	:
For: GENETIC SEQUENCES ENCODING SUBSTRATE-SPECIFIC DIHYDROFLAVANOL 4-REDUCTASE AND USES THEREFOR	:

Assistant Commissioner of Patents
Washington, D.C. 20231

SIR:

PRELIMINARY AMENDMENT

Prior to examination of this application please make the enter the following amendments.

In the claims:

Cancel claims 1-21
Add new claims 22-26

--22. (new) 1. A method for producing a plant having a phenotype characterized by an increased production of pelargonidin derivatives comprising the steps of:

(i) isolating a first nucleic acid according to SEQ ID NO: 1 encoding a dihydroflavanol-4-reductase;

(ii) carrying out site specific mutagenesis on said first nucleic acid to produce a second nucleic acid wherein the codon for the Asn residue at position 134 has been mutated to a codon for Leu.

(iii) introducing said second nucleic acid into a vector wherein said second nucleic acid is operably linked to a promoter;

(iv) transforming a plant cell with said vector;

(v) regenerating plants from one or more of said transformed plant cells;

(vi) selecting a plant with the desired phenotype.

2. A method as recited in claim 1 wherein said first nucleic acid is from *Gerbera.*

3. A method as recited in claim 1 wherein said promoter is a cauliflower mosaic virus promoter.

4. A method as recited in claim 1 wherein said promoter is an inducible promoter.

5. A method as recited in claim 1 wherein said promoter is a tissue specific promoter.--

In the specification:

In the specification following the title but before the first line of text, insert the following:

-- *Cross-Reference to Related Applications*

This is a divisional of Serial No. 09/638,715 , currently pending. --

On page 3, lines 5-7, please amend the specification as follows:

--Accordingly, the object of this invention is to provide substrate-specific *DFR*s which have [altered] point mutations at residue number 134 of SEQ ID NO: 2 when the amino acids [sequences at the substrate specificity determining region] are aligned with the ClustalW program.--

On page 7, lines 7, please amend the specification as follows:

--using cDNA sequences of *Petunia* (SEQ ID NO: 35) and *Gerbera* (SEQ ID NO: 1).--

On page 13 after "Chimeric gene construction" please replace the present paragraph with the following paragraph:

--Highly conserved regions of the *DFR* gene were identified by a multiple sequence alignment of a number of DFRs. The 5' region (*Gerbera DFR* portion) of each chimeric gene was synthesized from the *Gerbera DFR* cDNA clone using a primer containing the codon for the starting methionine of the *Gerbera DFR* gene (SEQ ID NO. 5): [(]5'-GGC GAA AAT GGA AGA GGA TTC TCC-3'[)] and a primer containing a conserved region of the *Gerbera DFR* gene (Chimera 1; SEQ ID NO: 6: 5'-AGC AGA TGA AGT GAA CAC TAG TTT CTT CAC-3'; Chimera 2; SEQ ID NO: 7: 5'-GGC TTT CTC TGC

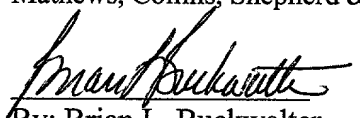
CAG AGT TTT TGA CAC GAA-3'; Chimera 3; SEQ ID NO: 8: 5'-GTG GGA CGA GCA AAT GTA TCT TCC TTT TGC-3 '). The 3' region *(Petunia DFR* portion) of each chimeric gene was synthesized from the *Petunia DFRA* cDNA clone using a primer complementary to the three conserved regions (Chimera 1; SEQ ID NO: 9: 5'- TTC ACT TCA TCT GCT GGA ACT CTC GAT GTG; Chimera 2; SEQ ID NO: 10: 5'-CTG GCA GAG AAA GCC GCA ATG GAA GAA GCT-3'; Chimera 3; SEQ ID NO: 11: 5'-ATT TGC TCG TCC CAC CAT GCT ATC ATC TAC-3') and a primer containing the stop codon of the *Petunia DFRA* gene (SEQ ID NO: 12): [(]5'-GCG CTA GAC TTC AAC ATT GCT T AA-3 '[)]. 5' and 3' regions were gel purified after PCR amplification. To assemble the full length chimeric gene the 5' and 3' region fragments were added to the same tube in roughly equal amounts and subjected to PCR cycles (94°C 30", 55°C 30", 72°C 1 :30). Full-length chimeric genes (-1.1 kb) were purified from agarose gels. The chimeric genes were cloned into a vector containing the 35S CaMV promoter and NOS terminator. *Pfu* polymerase (Stratagene, La Jolla, CA) was used for all PCR reactions.--

REMARKS

The present case is a Divisional Application of Ser No.09/638,715. The original claims 1-22 have been canceled and new claims 22-25 are pending. Claims 22-25 correspond to the claims in Group III (Claims 18-20) of the parent case.

The specification as amended complies with requirements for a sequence listing under 1.821-1.825. A paper copy of the sequence listing is enclosed. A request under 1.821(e) to use the computer readable disk from the parent case accompanies this application. A copy of the declaration and power of attorney from the parent case has been enclosed as provided by 37 C.F.R. § 1.64(d)(1)(ii).

Respectfully submitted,
Mathews, Collins, Shepherd & McKay, P.A.



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APPENDIX

CLEAN COPY OF CLAIMS

22. A method for producing a plant having a phenotype characterized by an increased production of pelargonidin derivatives comprising the steps of:

    (i) isolating a first nucleic acid according to SEQ ID NO: 1 encoding a dihydroflavanol-4-reductase;

    (ii) carrying out site specific mutagenesis on said first nucleic acid to produce a second nucleic acid wherein the codon for the Asn residue at position 134 has been mutated to a codon for Leu.

    (iii) introducing said second nucleic acid into a vector wherein said second nucleic acid is operably linked to a promoter;

    (iv) transforming a plant cell with said vector;

    (v) regenerating the plants from one or more of said transformed plant cells;

    (vi) selecting a plant with the desired phenotype.

23. A method as recited in claim 1 wherein said first nucleic acid is from *Gerbera*.

23. A method as recited in claim 1 wherein said promoter is a cauliflower mosaic virus promoter.

24. A method as recited in claim 1 wherein said promoter is an inducible promoter.

25. A method as recited in claim 1 wherein said promoter is a tissue specific promoter.--

CLEAN COPY OF AMENDED SPECIFICATION

On page 3, lines 5-7:

Accordingly, the object of this invention is to provide substrate-specific *DFR*s which have point mutations at residue number 134 of SEQ ID NO: 2 when the amino acids are aligned with the ClustalW program.

On page 7, first paragraph:

In accordance with the present invention, the substrate specificity determining region was identified by determining the abilities of three chimeric *DFRs* to catalyze the reduction of DHK in the transgenic *Petunia* lines. In order to identify the region of DFR that determines its substrate specificity, we constructed chimeric *DFR* genes using cDNA sequences of *Petunia* (SEQ ID NO: 35) and *Gerbera* (SEQ ID NO: 1). Though these two DFRs have high similiarity at the amino acid level, *Gerbera* DFR is able to catalyze dihydrokaempferol (DHK) while *Petunia* DFR cannot (Elomaa et al. Mol. Gen. Genet. 248:649-656 (1995)). We built three different chimeric genes using regions of high homology as common PCR primer sites (Fig1A). The chimeric genes were transformed into a white flowered *Petunia* mutant (W80) that lacks DFR activity and accumulates primarily DHK but with appreciable amounts of dihydroquercetin (DHQ) and dihydromyricetin (DHM) (Huits et al., 1994). Chimera 1 produced pink flowers while Chimeras 2 and 3 bore orange-pink flowers (Fig. 1B). The hue of Chimera 1 flowers is very similar to the inbred *Petunia* mutant RLO1, which has functional DFR activity and accumulates DHK. Thin layer chromatography (TLC) determined that Chimera 1 produced mainly cyanidin and delphinidin (Fig. 1b). Chimeras 2 and 3 primarily produced pelargonidin (Fig. 1 C), which is the downstream product of DFR reduction of DHK. These results indicated that the region of DFR conferring the ability to reduce DHK was between Chimeras 1 and 2. The identified region (approx. 40 amino acids) is highly variable in DFRs from different plant species. By excluding the completely conserved amino acid sequences at the borders, the identified region is narrowed down to 26 amino acids. Hereinafter, this region is referred as substrate specificity determining

region. An example of the substrate specificity determining region in a few representaive DFRs is shown in Figure 2.

On page 13:

Chimeric gene construction

Highly conserved regions of the *DFR* gene were identified by a multiple sequence alignment of a number of DFRs. The 5' region (*Gerbera DFR* portion) of each chimeric gene was synthesized from the *Gerbera DFR* cDNA clone using a primer containing the codon for the starting methionine of the *Gerbera DFR* gene (SEQ ID NO. 5): 5'-GGC GAA AAT GGA AGA GGA TTC TCC-3' and a primer containing a conserved region of the *Gerbera DFR* gene (Chimera 1; SEQ ID NO: 6: 5'-AGC AGA TGA AGT GAA CAC TAG TTT CTT CAC-3'; Chimera 2; SEQ ID NO: 7: 5'-GGC TTT CTC TGC CAG AGT TTT TGA CAC GAA-3'; Chimera 3; SEQ ID NO: 8: 5'-GTG GGA CGA GCA AAT GTA TCT TCC TTT TGC-3 '). The 3' region *(Petunia DFR* portion) of each chimeric gene was synthesized from the *Petunia DFRA* cDNA clone using a primer complementary to the three conserved regions (Chimera 1; SEQ ID NO: 9: 5'- TTC ACT TCA TCT GCT GGA ACT CTC GAT GTG; Chimera 2; SEQ ID NO: 10: 5'-CTG GCA GAG AAA GCC GCA ATG GAA GAA GCT-3'; Chimera 3; SEQ ID NO: 11: 5'-ATT TGC TCG TCC CAC CAT GCT ATC ATC TAC-3') and a primer containing the stop codon of the *Petunia DFRA* gene (SEQ ID NO: 12): 5'-GCG CTA GAC TTC AAC ATT GCT T AA-3 '. 5' and 3' regions were gel purified after PCR amplification. To assemble the full length chimeric gene the 5' and 3' region fragments were added to the same tube in roughly equal amounts and subjected to PCR cycles (94°C 30", 55°C 30", 72°C 1 :30). Full-length chimeric genes (-1.1 kb) were purified from agarose gels. The chimeric genes were cloned into a vector containing the 35S CaMV promoter and NOS terminator. *Pfu* polymerase (Stratagene, La Jolla, CA) was used for all PCR reactions.